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Comparative Genomics of Atypical Enteropathogenic *Escherichia coli* from Kittens and Children Identifies Bacterial Factors Associated with Virulence in Kittens

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ABSTRACT Typical enteropathogenic *Escherichia coli* (tEPEC) is a leading cause of diarrhea and associated death in children worldwide. Atypical EPEC (aEPEC) lacks the plasmid encoding bundle-forming pili and is considered less virulent, but the molecular mechanism of virulence is poorly understood. We recently identified kittens as a host for aEPEC where intestinal epithelial colonization was associated with diarrheal disease and death. The purposes of this study were to (i) determine the genomic similarity between kitten aEPEC and human aEPEC isolates and (ii) identify genotypic or phenotypic traits associated with virulence in kitten aEPEC. We observed no differences between kitten and human aEPEC in core genome content or gene cluster sequence identities, and no distinguishing genomic content was observed between aEPEC isolates from kittens with nonclinical colonization (NC) versus those with lethal infection (LI). Variation in adherence patterns and ability to aggregate actin in cultured cells mirrored descriptions of human aEPEC. The aEPEC isolated from kittens with LI were significantly more motile than isolates from kittens with NC. Kittens may serve as a reservoir for aEPEC that is indistinguishable from human aEPEC isolates and may provide a needed comparative animal model for the study of aEPEC pathogenesis. Motility seems to be an important factor in pathogenesis of LI associated with aEPEC in kittens.

KEYWORDS enteropathogenic *E. coli*, EPEC, animal model, phylogenetic analysis

Diarrhea is responsible for the death of an estimated 500,000 children per year worldwide, with the majority of cases of mortality occurring in developing countries (1, 2). Recent studies have identified enteropathogenic *Escherichia coli* (EPEC) as a leading cause of diarrhea in these children (3, 4). In the recent Global Enteric Multicenter Study (GEMS), the EPEC isolates were identified as a significant cause of childhood morbidity and mortality (3). Diarrhea caused by EPEC infection afflicts approximately 17 million children each year (1), resulting in >120,000 fatalities (1, 4).

Enteropathogenic *E. coli* is an attaching and effacing pathogen that adheres to intestinal epithelial cells and alters their function. In typical strains of EPEC (tEPEC), virulence is promoted by the presence of a plasmid harboring a bundle-forming pilus gene cluster (*bfp*), which promotes initial adherence of the isolate to epithelial cells (3). Tight adherence of EPEC is subsequently mediated by intimin, an adhesin encoded by the *E. coli* attaching and effacing (*eae*) gene (5). The receptor for intimin, Tir, along with other bacterial effector proteins, is injected by EPEC into host cells by using the type 3 secretion system (T3SS). These effector proteins lead to increased intestinal permeability, altered electrolyte transport, and gut malabsorption (6–15). In contrast to tEPEC,

Citation Watson VE, Hazen TH, Rasko DA, Jacob ME, Elfenbein JR, Stauffer SH, Gookin JL. 2021. Comparative genomics of atypical enteropathogenic *Escherichia coli* from kittens and children identifies bacterial factors associated with virulence in kittens. Infect Immun 89:e00619-20. https://doi.org/10.1128/IAI.00619-20.

Editor Igor E. Brodsky, University of Pennsylvania

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For a commentary on this article, see https://doi.org/10.1128/IAI.00752-20.

Received 8 October 2020
Accepted 11 November 2020
Accepted manuscript posted online 30 November 2020
Published 16 February 2021

TABLE 1 Isolate and genome characteristics of the aEPEC from kittens

	Isolate			Assembly					Genome					
Isolate no.	Clinical outcome	Kitten	Serotype	No. of contigs	Genome size (bp)	GC content (%)	N_{50}	GenBank accession no.	*In silico* serotype	*In silico* MLST	Phylogroup	EPEC lineage	AMR genes	Plasmid(s) (GenBank accession)
K5-1	NC	K5	O88:H-	209	5,378,008	50.44	75,797	VXDG00000000	O88:H8	ST590	B1	None	None	IncFIA, IncFIB (AP001918), IncFII (pCoo), IncFII (pHN7A8), IncI2, IncY
K15-1	NC	K15	O128:H2	218	5,195,297	50.60	75,218	VXDH00000000	O128ab:H2	ST20	B1	EPEC2	None	Col440II, IncFII(pCoo), IncX1
12-1	NC	12	O153:H7	145	5,027,534	50.49	123,651	VXDI00000000	O153/O178:H7	ST328	B1	EPEC7	None	IncFII(pCoo)
12-3	NC	12	O128:H2	175	5,114,800	50.66	75,031	VXDJ00000000	O128ab:H2	ST20	B1	EPEC2	None	IncFIA, IncFIB (AP001918), IncFII (pCoo)
25-2	NC	25	O128:H2	210	5,184,882	50.56	63,848	VXDK00000000	O128ab:H2	ST20	B1	EPEC2	None	IncFIA, IncFIB (AP001918), IncFII (pCoo)
55-3	NC	55	O153:H-	142	4,939,863	50.58	124,911	VXDL00000000	OND:H21	ST40	B1	None	None	None
5-1	LI	5	O108:H21	88	5,101,464	50.5	186,955	VXDM00000000	O108:H21	ST337	B1	None	None	IncFIB(AP001918), IncFII
31-1	LI	31	O153:H21	96	5,005,717	50.66	107,349	VXDN00000000	OND:H21	ST40	B1	None	None	IncFIA, IncFIB (AP001918), IncFII (pCoo), IncFII (pRSB107)
33-1	LI	33	O153:H7						NA[a]					
33-2	LI	33	O111:H8	329	5,142,983	50.52	52,872	VXDO00000000	O111:H8	ND[b]	B1	None	None	Col156, Col440II, IncFIB (AP001918)
34-1	LI	34	O153:H7	167	5,188,103	50.55	91,429	VXDP00000000	O153/O178:H7	ST328	B1	EPEC7	None	IncFIA, IncFIB (AP001918), IncFII (AY458016), IncFII (pCoo)
35-2	LI	35	O4:H+						NA					
35-4	LI	35	O153:H7	155	5,106,488	50.51	128,753	VXDQ00000000	O153/O178:H7	ST328	B1	EPEC7	None	IncFIA, IncFIB (AP001918), IncFII (pCoo)
53-1	LI	53	O153:H21	117	5,201,442	50.51	113,480	VXDR00000000	OND:H21	ST40	B1	None	bla_{TEM-1}, *dfrA12*, *aadA2*, *mphA*, *sul1*	IncFIA, IncFIA(HI1), IncFIB(AP001918), IncHI1A, IncHI1B (R27), IncI1, IncQ1

[a]NA, not applicable for the two kitten aEPEC isolates that assembled poorly and were excluded from further genomic analyses; however, these isolates were included in other functional assays in this study.
[b]ND, the *in silico* MLST could not be determined.

atypical EPEC (aEPEC) is less virulent due in part to lack of the *bfp* gene cluster. The factors promoting the initial adherence of aEPEC to epithelial cells and significance of aEPEC in childhood diarrhea are less well understood. Atypical EPEC is associated with prolonged diarrhea in some studies (16, 17) but can be observed in children with and without diarrhea (18, 19). In an effort to better understand the virulence of EPEC, recent phylogenetic studies have identified that childhood isolates are highly diverse and that multiple genes and gene clusters are associated with increased severity of disease (20–26).

Similar to that in children, death associated with diarrheal disease is common in young food-producing and companion animals in the United States (27–29). The association of aEPEC with diarrhea in these species is also unclear, as is their speculated potential to serve as a reservoir for human infection (30–33). In investigating causes of death in orphan kittens, a companion animal population afflicted with a high rate of diarrhea and mortality, we observed a significant number of animals with postmortem diagnosis of attaching and effacing *E. coli* infection (29). The culture-based prevalence of aEPEC infection in kittens and detection in both normal kittens and kittens with diarrhea (34) mirrors that described in children with aEPEC (35). However, the onset and duration of diarrhea in kittens with aEPEC is not as well characterized in this population. Kittens with aEPEC and diarrhea had a significantly higher quantity (CFU/gram) of fecal aEPEC, more severe intestinal pathology, need for parenteral fluid administration, and mortality compared to kittens with diarrhea that did not have aEPEC (34). These findings suggest that aEPEC infection in kittens mimics that of aEPEC in children.

Here, we conducted a comparative analysis of aEPEC isolates from healthy kittens and those with diarrhea-related mortality. We determined the genomic similarity between kitten aEPEC and human aEPEC isolates. Furthermore, we determined phenotypic traits associated with virulence. This study provides an improved understanding of the pathogenesis of aEPEC infection and highlights the similarities between human and kitten aEPEC isolates, suggesting translational potential of kittens for understanding human aEPEC disease.

RESULTS

Source, identification, and selection of kitten aEPEC isolates. A total of 25 isolates of kitten aEPEC were previously obtained from feces from six orphaned kittens that died or were euthanized due to severe diarrhea (lethal infection [LI], 17 isolates) (34), three healthy orphaned kittens euthanized due to overpopulation (six isolates) (34), and two healthy kittens purchased from a commercial vendor (two isolates) (nonclinical colonization [NC]) (36). Each isolate was identified as atypical (aEPEC) based on the presence of *eae* and absence of the genes encoding bundle forming pilus (BFP) and Shiga toxins 1 and 2. All isolates also lacked genes encoding heat-stable and heat-labile toxins (*estA*, *eltA*, and *eltB*) from enterotoxigenic *E. coli*, invasion plasmid antigen H (*ipaH*) from *Shigella*, enteroaggregative plasmid pCVD432 from enteroaggregative *E. coli*, and cytotoxic necrotizing factors 1 and 2 (*cnf1* and *cnf2*) from extraintestinal pathogenic *E. coli*. All isolates were further confirmed to be aEPEC on the basis of whole-genome sequencing. For each kitten from which multiple aEPEC isolates were obtained, unique aEPEC isolates were selected on the basis of results of serotype and pulsotype as previously described and reported (34, 36). This yielded six unique isolates from kittens with nonclinical colonization and eight unique isolates from kittens with lethal infection as shown in Table 1.

Genome sequencing and phylogenomic analysis of kitten aEPEC with comparison to human EPEC. To characterize the genomic diversity of kitten aEPEC, we sequenced the genomes of 14 kitten aEPEC isolates, including six from nonclinical colonization and eight from lethal infection outcomes (Table 1). Of the 14 kitten aEPEC isolates, two assembled poorly following genome sequencing, and these two assemblies were excluded from further genomic analyses (Table 1). Of the remaining 12 kitten aEPEC genome assemblies, the average genome assembly size was 5,132,215 bp (range, 4,939,863 to 5,378,008 bp) and average GC content was 50.55% (range, 50.44% to

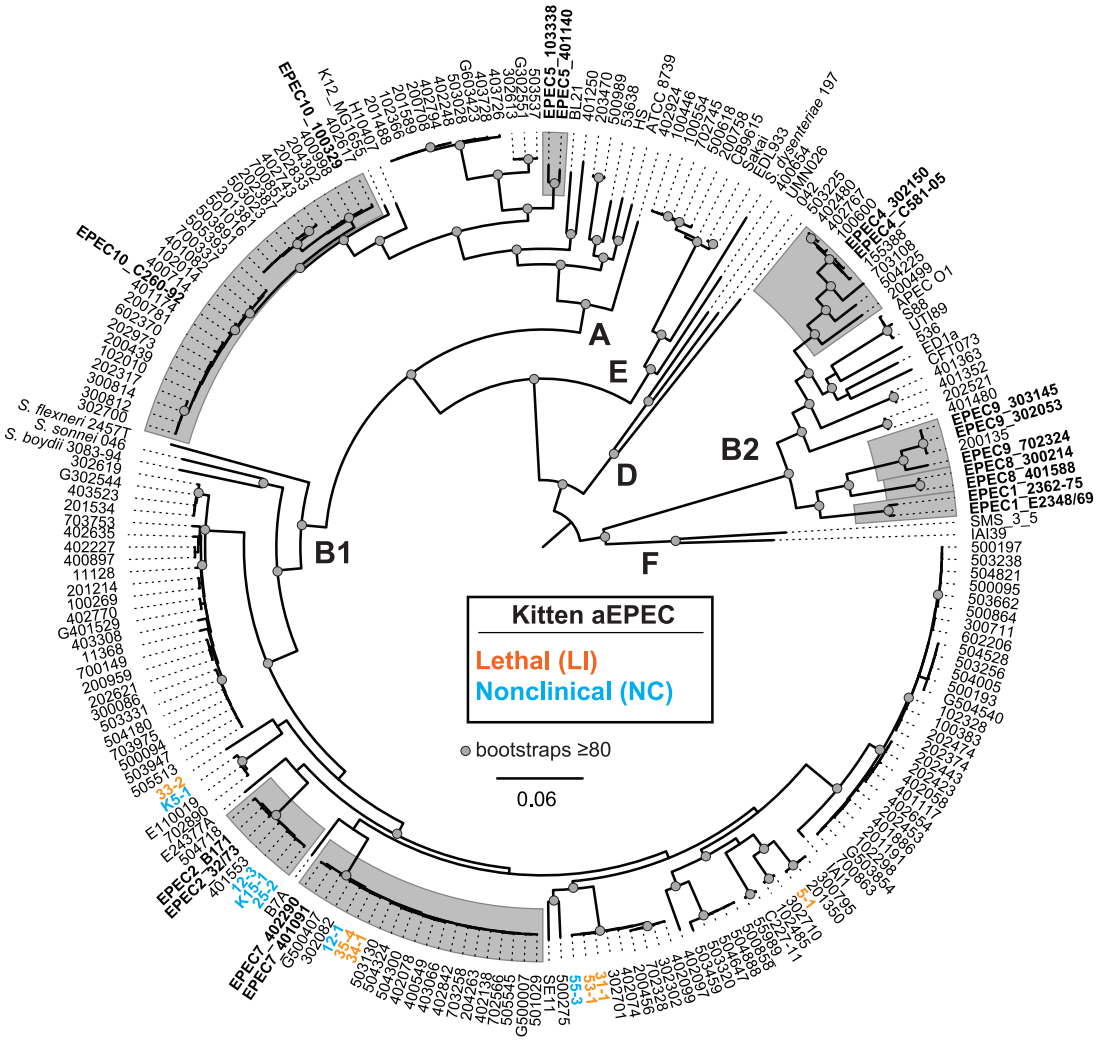

FIG 1 Phylogenomic analysis of 12 kitten aEPEC isolates associated with nonclinical colonization (NC) or lethal (LI) infections compared with a previously sequenced collection of 149 aEPEC genomes and 49 diverse *E. coli* and *Shigella* reference genomes. The kitten aEPEC genomes are indicated by blue (NC) and orange (LI) labels. The previously described EPEC phylogenomic lineages (22, 38) are indicated with gray shading, and the lineage reference genomes are in boldface font. The *E. coli* and *Shigella* phylogroups (A, B1, B2, D, E, and F) are indicated in the interior of the phylogeny. Bootstrap values ≥80 are indicated by gray circles. The scale bar indicates the approximate distance of 0.06 changes per site.

50.66%) (Table 1). *In silico* prediction of the serotypes of each of the kitten aEPEC genomes identified six different serotypes, which were largely consistent with the laboratory-based serotype determinations (Table 1). Also, *in silico* prediction of the multilocus sequence types (MLSTs) identified at least six sequence types (STs), with ST20 predicted for three of the six nonclinical colonization kitten aEPEC isolates (NC), and ST328 and ST40 each predicted for two of the lethal kitten aEPECs (LI) (Table 1). Detection of known antimicrobial resistance genes demonstrated that only one of the kitten aEPEC genomes contained resistance genes other than intrinsic efflux pumps. LI isolate 53-1 contained predicted $bla_{\text{TEM-1}}$, *dfrA12*, *aadA2*, *mphA*, and *sul1* genes (Table 1). In addition, detection of plasmid incompatibility types in each of the kitten aEPEC genomes demonstrated that all but one of the genomes contained at least one region with similarity to a known plasmid family (Table 1). These predicted plasmid types include IncFII and IncFIB plasmid families, which count many of the *E. coli* virulence plasmids among their members (37).

We also compared the genomic similarity of the 12 kitten aEPECs to a collection of 149 previously characterized aEPECs from humans (22), using both phylogenomic and

TABLE 2 Number of gene clusters identified using LS-BSR in relation to species of origin (cat versus human) and clinical outcome (LI versus NC)

		No. of genomes		No. of LS-BSR gene clusters[a]		
Group 1	Group 2	Group 1	Group 2	All genomes	≥50% of genomes	≥1 genome
LI (cat)	NC (cat)	6	6	0	23	863
NC (cat)	LI (cat)	6	6	0	265	1,010
Cat (LI+NC)	Human	12	151	0	4	314
Human	Cat (LI+NC)	151	12	0	16	8,430

[a]The total number of core gene clusters (LS-BSR ≥ 0.8) in all of the genomes ($n = 163$) analyzed was 3,321. The number of gene clusters that were present in all genomes, ≥50% of the genomes, or ≥1 of the genomes of group 1 (LS-BSR ≥ 0.8) and absent from all of the genomes of group 2 (LS-BSR < 0.4).

gene-based approaches. Interestingly, phylogenomic analysis demonstrated that all of the kitten aEPEC genomes were identified in phylogroup B1, whereas the human aEPECs were identified in phylogroups A, B1, B2, D, and E (Fig. 1; Table 1). However, additional genome sequencing of kitten aEPECs may identify isolates that belong to the other diverse *E. coli* phylogroups. By including reference genomes of the previously described tEPEC phylogenomic lineages (21, 38), we determined that three of the non-clinical colonization (NC) kitten aEPECs were identified in the EPEC2 lineage and one NC aEPEC was identified in the EPEC7 lineage, while the remaining three NC aEPECs were not in a previously defined tEPEC lineage (Fig. 1; Table 1). Of the six lethal infection (LI) kitten aEPEC genomes sequenced, two were identified in the EPEC7 lineage, while the remaining four LI kitten aEPEC genomes were not in previously defined tEPEC lineages (Fig. 1; Table 1).

Using large-scale BLAST score ratio (LS-BSR) to analyze the total genome content of selected human aEPEC isolates and all kitten aEPEC isolates, no gene clusters were identified as being present in all of the kitten aEPECs and absent from all of the human aEPECs, or vice versa (Table 2). Likewise, no genome clusters were identified that differentiated all of the NC versus LI kitten aEPEC isolates (Table 2). There were 23 gene clusters that were identified in ≥50% of the LI kitten aEPEC genomes that were absent from all of the NC kitten aEPECs, compared to 265 gene clusters that were present in ≥50% of the NC kitten aEPECs that were absent from all of the LI kitten aEPEC genomes (Table 2 and see Table S1 in the supplemental material). Interestingly, many of the genes associated with the NC kitten aEPEC had predicted protein functions associated with phage (Table S1). This finding suggests there is greater gene conservation among the NC kitten aEPEC isolates than with the LI kitten aEPECs; however, further analyses of a larger number of genomes would be needed to determine if particular aEPEC isolates and/or genes are more associated with NC versus LI outcomes in kittens. Also, there were only four gene clusters identified in ≥50% of the kitten aEPEC genomes that were absent from all of the human aEPEC genomes, and only 16 gene clusters identified in ≥50% of the human aEPECs that were absent from all kitten aEPECs (Table 2 and S1). These data suggest that there are no genomic differences that are exclusive to all of the isolates from either source. The greatest number of gene clusters identified as being unique for the kitten versus human aEPECs, or NC versus LI kitten aEPECs, were isolate specific, as many were identified in a single genome (Table 2). Overall, the gene-based comparisons indicate that the kitten aEPEC isolates are indistinguishable on the genomic level from human aEPEC isolates occupying the same EPEC phylogenomic lineage.

Virulence genes associated with kitten aEPEC from different clinical outcomes. To establish whether conserved sequences of particular virulence genes were associated with NC versus LI outcomes in kitten aEPEC isolates, we characterized a subset of known virulence genes bioinformatically using BSR (Fig. 2) and by PCR for select locus of enterocyte effacement (LEE) genes (*escN*, *ler*, and *espA*) and the adherence-

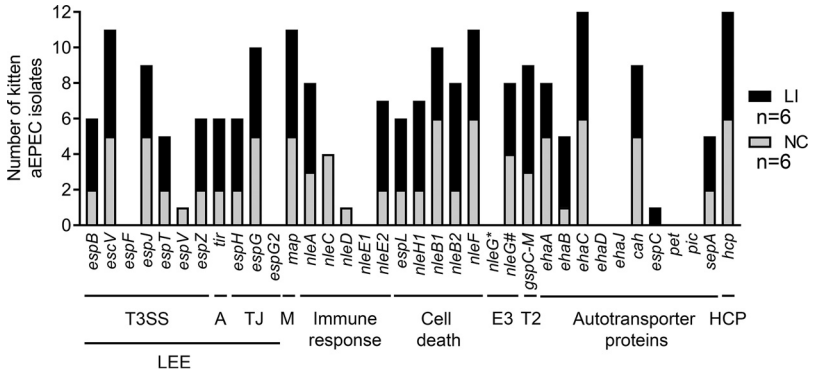

FIG 2 Presence in kitten aEPECs of *E. coli* genes of potential association with virulence. The presence of each virulence gene was determined using BSR values of ≥0.8 indicating high similarity to the reference strains of EPEC. Each bar represents the number of LI (black) isolates and number of NC (gray) isolates of aEPEC. The total number of kitten aEPECs ($n = 12$) with conserved virulence genes is shown on the *y* axis. Effect of each gene or defining characteristic of the genes are given along the *x* axis. T3SS, type 3 secretion system; *esp*, *E. coli* secreted protein; A, actin pedestal formation; TJ, tight junction disruptors; LEE, locus of enterocyte effacement; M, mitochondrial dysfunction; E3, E3 ubiquitin ligase; T2, type 2 secretion system genes; HCP, hemorrhagic coli pilus.

associated gene *efa1* (*lifA*) (Table 3). Neither BSR nor PCR demonstrated significant differences in the percentage of NC versus that of LI isolates having conserved sequences for these individual genes.

Phenotypic characterization of kitten aEPEC from different clinical outcomes. All 14 kitten aEPEC isolates (Table 1), including six from NC and 8 from LI outcomes, were assayed for biofilm formation, motility, and adherence to HEp-2 cells *in vitro*. Compared to a known non-biofilm-forming isolate of tEPEC, the majority of kitten aEPEC isolates were identified as biofilm formers. As expected, the presence of salt in the medium reduced biofilm formation for all isolates (Fig. 3). No significant difference in the magnitude of biofilm formation was observed between aEPEC isolates obtained from NC versus from LI kittens.

With the exception of one NC isolate, all isolates of aEPEC were determined to be motile (Fig. 4). The NC isolates of aEPEC had an average (± standard error of the mean [SEM]) motility of 8.1 ± 0.8 mm, while the LI isolates of aEPEC had an average motility of 12.4 ± 1.7 mm. The motility of LI isolates was significantly greater than that of NC isolates of kitten aEPEC ($P < 0.05$, Student's *t* test).

Among the 14 aEPEC isolates, 13 (93%) were demonstrated to adhere to cultured HEp-2 cells based on results of a Romanowsky stain. Patterns of adherence included localized adherence like (LAL), diffuse adherence (DA), and adherence of isolated individual bacteria (IS) as previously described for human aEPEC (39). Fewer isolates (6/14 [43%]) demonstrated a range in intensity of recruitment of actin to sites of bacterial attachment based on results of the fluorescence actin-staining (FAS) assay (Fig. 5 and Table S2). There was no significant difference between LI and NC isolates in attachment to HEp-2 cells or ability to recruit actin.

TABLE 3 Presence of conserved locus of enterocyte effacement genes, on the basis of PCR amplification, in 14 kitten aEPEC isolates

	No. (%) of isolates		
Virulence gene	**aEPEC ($n = 14$)**	**NC ($n = 6$)**	**LI ($n = 8$)**
escN	10 (71)	3 (50)	7 (88)
ler	10 (71)	3 (50)	7 (88)
espA	6 (43)	3 (50)	3 (38)
efa1/lifA	5 (36)	1 (17)	4 (50)

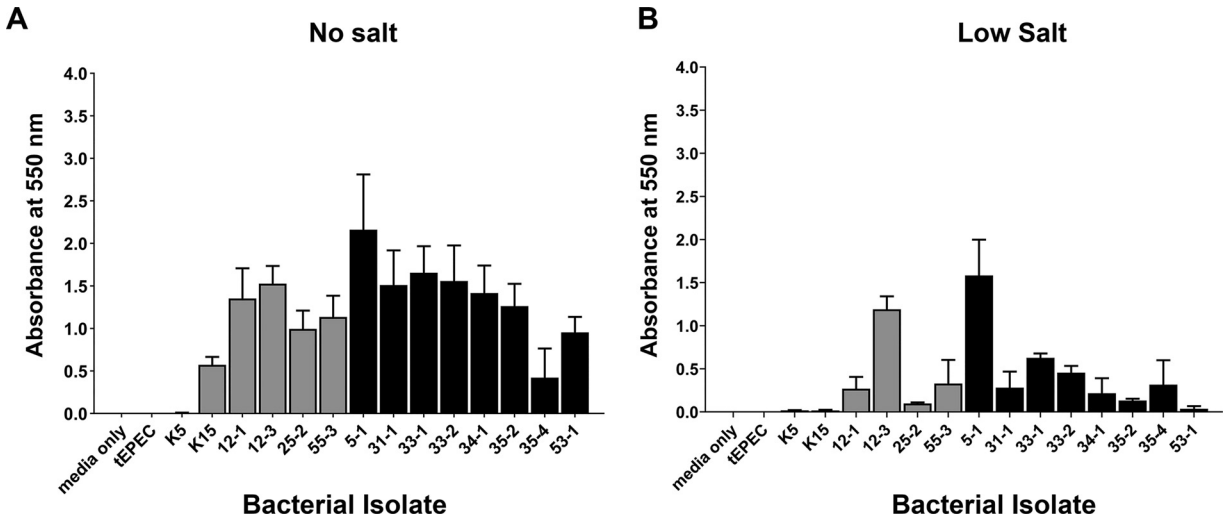

FIG 3 B Biofilm formation by aEPEC isolates from kittens with nonclinical colonization (NC; gray bars) and lethal infection (LI; black bars). Graph demonstrates the absorbance of crystal violet at 550 nm following biofilm formation by designated kitten aEPEC isolates cultured in no-salt LB medium (A) and low-salt LB medium (B).

DISCUSSION

In this study, we performed a core genome phylogenetic analysis of 12 aEPEC isolates from lethal and nonclinical colonized cases of diarrheal illness in orphaned kittens with comparison to isolates of human aEPECs, including all sequences of isolates obtained from children enrolled in the Global Enteric Multicenter Study (3). The results establish that kitten aEPECs possess considerable genomic diversity and cannot be distinguished phylogenetically, or by total gene content, from the genomes of aEPECs from humans or other sources (21, 22, 40, 41). This observation supports the presumption that nonhuman species, in this case, cats, may serve as a reservoir for human aEPEC exposure (32, 42).

In an attempt to define a more virulent genome of kitten aEPEC, we compared the gene content of aEPEC isolates from nonclinical colonized kittens to those from kittens with lethal diarrhea. Among these isolates, no gene clusters were exclusively associated with infection outcome. When applied to childhood isolates of EPECs, these methods have identified several genes and gene clusters within the genome that are associ-

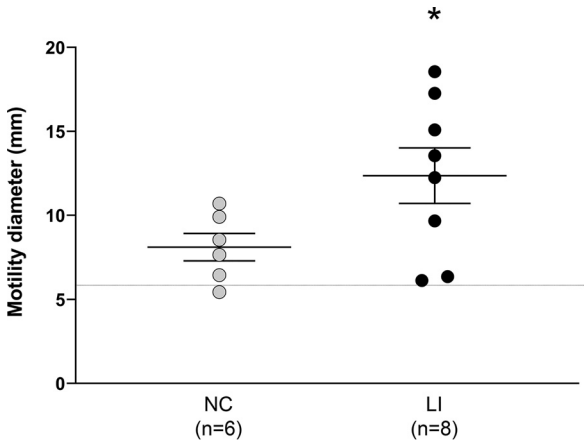

FIG 4 Assay of motility by kitten aEPEC. Each data point represents the mean diameter of motility of a single isolate determined from repeated motility assays. The horizontal lines indicate the means and SEMs for isolates from nonclinical colonization (NC; gray circles) and lethal infection (LI; black circles). Gray line represents assigned threshold for designation of motility. *, $P = 0.03$; Student's *t* test.

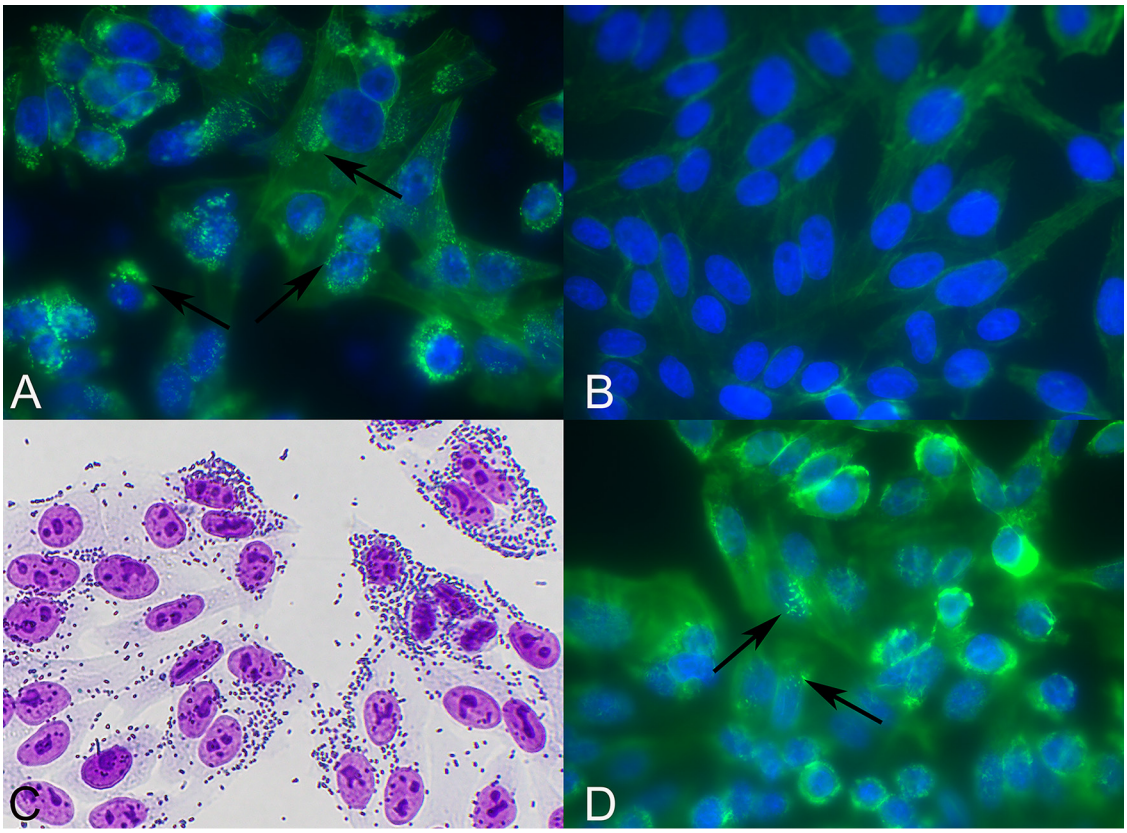

FIG 5 Fluorescence actin and conventional eosin and methylene blue staining of HEp-2 cell monolayers infected with kitten aEPEC isolates. (A) FAS-positive control monolayers infected with tEPEC strain E2348/69 showing a localized adherence (LA) pattern. (B) FAS-negative kitten aEPEC (isolate 35-2). (C) Conventional eosin and methylene blue stain of monolayers infected with kitten aEPEC (isolate K5) showing a hybrid localized adherence-like (LAL) and diffuse adherence (DA) pattern. (D) FAS-positive adherence of bacteria from the same isolate shown in panel C showing an LAL adherence pattern. Arrows indicate actin filaments (green) beneath adherent bacteria. For FAS assay, monolayers were counterstained with the nuclear stain DAPI (blue).

ated with increased severity of disease, including presence of the EAF plasmid (i.e., *bfp* positive) that is characteristic of tEPEC strains (20–23, 38, 43–45). All kitten isolates were *bfp* negative, which explains why this association was not observed. Additional gene clusters associated with symptomatic outcomes of infection in childhood EPEC include alleles of the T3SS effectors NleG and EspJ, presence of the OI-122 pathogenicity island, and other unknown and phage-associated genes (20, 21, 24–26). Using targeted PCR, conserved sequences of genes spanning the LEE were amplified from the majority of kitten aEPEC isolates. It is worthwhile to note that no genes of aEPEC have yet been identified as universally associated with the presence of clinical disease. This fact, along with the greater genomic diversity of aEPEC and relatively small number of kitten isolates examined, likely explains why specific disease-associated genes were not identified in the present study. Another important consideration is that the pathogenicity of aEPEC is also dependent on host susceptibility and not simply on the presence of bacterial virulence factors. For example, we have previously shown that healthy kittens do not develop diarrhea following experimental infection by kitten aEPEC, while kittens with disruption of their intestinal microbiota prior to experimental infection experience increased water losses via their feces (36).

A key asset of the present study is the combination of the genomic analysis with phenotypic characterization of aEPEC isolates from nonclinical colonized kittens compared to isolates from kittens with lethal diarrhea. We focused on phenotypic characteristics likely associated with increased exposure to aEPEC (biofilm formation) and capability of aEPEC to gain access to (motility) and colonize (adherence) intestinal

epithelial cells. There was no significant association between nonclinical colonization versus lethal isolates and magnitude of biofilm formation. However, aEPEC isolates from kittens with lethal diarrhea demonstrated significantly greater motility than isolates from kittens with nonclinical colonization, and in line with this finding, genes encoding flagellin are associated with symptomatic outcomes of infection in childhood EPEC (21). Moreover, we have previously demonstrated that kitten aEPEC isolates with flagellin type H21 are more commonly associated with diarrheal mortality (34). These observations support an association between motility of kitten aEPEC isolates and clinical disease outcome but do not prove a causal link.

We additionally demonstrated that kitten aEPECs adhere to HEp-2 cells in a variety of different patterns and not always accompanied by recruitment of host cell actin. Both of these findings closely mirror a substantial behavioral diversity described among human isolates of aEPEC in cell-based assays (39, 46) and support a potential for kitten aEPEC to infect human cells. While we did not observe an association between adherence or actin recruitment and diarrheal mortality, cell-based assays can be unreliable predictors of *in vivo* behavior or pathogenic potential (47). In support of this, our prior studies of the naturally occurring infection have identified adherence of aEPEC to the intestinal epithelium only in tissue samples from kittens with clinical illness (29, 36, 48).

The results of this study suggest that aEPEC isolates from kittens are genomically similar to those from human aEPEC infections. Our data suggest that motility of aEPEC may contribute significantly to disease severity in kittens. Further work is needed to establish the mechanism of increased motility and the relationship to disease severity for aEPEC isolates.

MATERIALS AND METHODS

Bacterial isolates. Isolates of aEPEC were previously cultured from feces (i) collected postmortem from apparently healthy kittens after euthanasia by an animal control facility, (ii) collected postmortem from kittens that died or were euthanized by an animal control facility due to severe diarrhea, and (iii) collected from live apparently healthy kittens purchased from a commercial vendor (34, 36). Fecal samples were obtained by swab of the colonic content. All sample collections were performed with approval from the North Carolina State University Institutional Animal Care and Use Committee. Colonic contents were swabbed and streaked onto MacConkey agar and incubated at 37°C for isolation of enteric bacteria. Bacteria were identified as *E. coli* based on presence of lactose fermentation and were streaked for purity on 5% Columbia sheep blood agar plates. Seven colonies per kitten were evaluated for the presence of indole reactivity and lack of pyrrolidonyl arylamidase and oxidase reactions using commercially available assays (Beckton, Dickinson and Company, Franklin Lakes, NJ). Isolates of *E. coli* were frozen in 50% glycerol-lysogeny broth (LB) at −80°C. Individual isolates of *E. coli* were evaluated by multiplex PCR (Pennsylvania State University *E. coli* Reference Center, University Park, PA) for the presence of canonical *E. coli* virulence genes encoding intimin (*eae*), Shiga toxins 1 and 2 (stx_1 and stx_2), heat-stable and heat-labile toxins (*estA*, *eltA*, and *eltB*), invasion plasmid antigen H (*ipaH*), enteroaggregative plasmid pCVD432, and cytotoxic necrotizing factors 1 and 2 (*cnf1* and *cnf2*) using published protocols (49, 50). Isolates positive for *eae* underwent conventional PCR for identification of bundle forming pilus (*bfp*) as previously described (51). Confirmation that *eae*-positive isolates were *E. coli* was performed using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) (Vitek MS, bioMérieux, Marcy l'Etoile, France) and verified by results of whole-genome sequencing.

Serotyping. Serotyping of the O antigen was performed using antisera directed to all identified O antigens (O1-O187) (52). H typing was performed by PCR amplification of the *fliC* (flagella) gene followed by analysis of HhaI restriction fragment length polymorphism (Pennsylvania State University *E. coli* Reference Center, University Park, PA) as previously described (53).

Genome sequencing and assembly. Atypical EPEC from kittens without clinical signs of diarrhea and aEPEC from kittens with clinical signs of diarrhea were selected for whole-genome sequencing (Table 1). Genomic DNA was isolated from each strain by growing a single colony overnight in LB medium in a shaking incubator set at 37°C. The genomic DNA was isolated using the GenElute genomic kit (Sigma-Aldrich, Corp., St. Louis, MO). Genome sequencing was performed using Illumina HiSeq 2500 with 150-bp paired-end reads. Following sequencing, the Illumina reads were subsampled to approximately 120× sequence coverage and were assembled using the modified Celera assembler MaSuRCA v.2.3.2 (54) with default parameters.

***In silico* prediction of serotypes, multilocus sequence types, antimicrobial resistance genes, and plasmid types.** The MLST sequence types (STs) of each of the kitten aEPEC genome assemblies were determined using the MLST scheme developed by Wirth et al. (55) using the MLST prediction tool of the Center for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/). Plasmids were

detected in the kitten aEPEC genome assemblies using PlasmidFinder v.1.3 (56) with the default 95% nucleotide identity threshold. The molecular serotype of each *E. coli* genome was predicted using SerotypeFinder v.2.0 (https://cge.cbs.dtu.dk/services/SerotypeFinder/) with the default settings of an 85% nucleotide identity threshold and 60% minimum alignment length (57). Antibiotic resistance genes were detected in each of the kitten aEPEC genome assemblies using the resistance gene identifier (RGI) and the comprehensive antibiotic resistance database (CARD) v.3.0.0, with perfect or strict identification criteria (58).

Phylogenomic analyses. The 12 kitten aEPEC genomes in this study were compared with 149 previously sequenced aEPEC genomes (22) and *E. coli* and *Shigella* reference genomes (see Table S3 in the supplemental material) by a single nucleotide polymorphism (SNP)-based phylogenomic analysis as previously described (21, 59, 60). *In Silico* Genotyper (ISG) was used to identify conserved SNP sites in all of the genomes relative to the genome of *E. coli* isolate IAI39 (GenBank accession number NC_011750.1) of phylogroup F as a reference. There were 159,427 conserved SNP sites used to infer a maximum likelihood phylogeny with RAxML v7.2.8 (61) with the GTR model of nucleotide substitution, the GAMMA model of rate heterogeneity, and 100 bootstrap replicates. The phylogeny was midpoint rooted and labeled using FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Gene alignments and phylogenetic analyses. Nucleotide sequences for specific genes were aligned in MEGA5 using the ClustalW algorithm as previously described (62, 63). A maximum likelihood phylogeny was constructed using the Kimura two-parameter model of distance estimation with 1,000 bootstrap replications (64).

BLAST score ratio analysis. The presence or absence of known virulence-associated genes in the genome sequences generated in this study was determined using BLAST score ratio (BSR) analysis with TBLASTN to compare the amino acid sequences of known virulence associated genes with each genome as previously described (21, 38, 44, 45). Protein-encoding genes that were considered present and with significant similarity had BSR values >0.8, while those with BSR values of <0.8 but ≥ 0.4 were considered to be present but divergent.

A *de novo* large-scale BLAST score ratio (LS-BSR) analysis was used to predict protein-encoding genes and compare their distributions using BLASTN among the 12 kitten aEPEC and 149 human aEPEC genomes, as previously described (21, 45). Genes that were identified with an LS-BSR value of ≥ 0.8 were considered present, while genes that had an LS-BSR value of <0.4 were considered absent from the genomes.

Virulence gene PCR. Each isolated colony of kitten aEPEC was mixed in 100 μl nuclease-free water and heated at 100°C for 30 min to liberate DNA. PCR amplification of virulence -associated genes spanning the locus of enterocyte effacement (LEE) were selected, including an ATPase of the T3SS (*escN*), an extracellular secreted protein (esp) associated with extension of the T3SS subunits (*espA*), and the LEE regulator (*ler*). Also selected for PCR was the non-LEE gene lymphocyte inhibitory factor (*lifA*) (otherwise known as enterohemorrhagic *E. coli* [EHEC] factor for adherence [*efa1*]) (26, 65–68). All conventional PCR assays were performed using previously published primer sequences and reaction conditions (68–71) (see Table S4 in the supplemental material). PCR products were isolated by electrophoresis on 1.5% agarose gels stained with either ethidium bromide or commercially available DNA stain (GelRed; Biotium, Fremont, CA) and visualized using a UV imager (Bio-Rad, Hercules, CA or UVP LLC, Upland, CA).

Biofilm formation. *In vitro* biofilm formation by each kitten aEPEC isolate was determined by incubation in 2 different growth media including lysogeny broth (LB) without salt and LB with low salt (5 g/liter NaCl). Biofilm assays were performed on 96-well polystyrene suspension culture plates (Olympus plastics; Genesee Scientific, San Diego, CA) as previously described (72). Briefly, overnight cultures of aEPEC were inoculated into wells containing medium at a 1:100 dilution and incubated statically at room temperature (~21°C) for 72 h. Media and planktonic bacteria were removed by rinsing 4 times, and plates were dried prior to adding 0.1% crystal violet for a 10-min incubation. Plates were rinsed 5 times to remove the crystal violet solution and dried overnight. Crystal violet retained within the biofilm was solubilized in 30% acetic acid for 10 min and quantified by absorption at 550 nm in a BioTek Synergy 2 microplate reader (BioTek US, Winooski, VT). Each experimental run included medium-only wells, typical EPEC (strain E2348/69) (73), nonpathogenic *E. coli* (ATTC 25922), and selected strains of kitten aEPEC. Each isolate was assayed in quadruplicates for each experiment and under each experimental condition, and each biofilm assay was repeated three times. Optical densities at 550 nm were averaged and compared for differences between NC and LI isolates by using a one-way analysis of variance (ANOVA).

Motility assay. Motility assays were performed as previously described (74, 75). Each aEPEC isolate was cultured overnight, diluted 100-fold, and normalized to an optical density at 600 nm (OD_{600}) using sterile phosphate-buffered saline (PBS). In addition to aEPEC isolates, tEPEC (E2348/69) and *E. coli* (ATTC 25922) were included in each assay as controls. Five microliters of each normalized aEPEC solution was inoculated onto 0.3% agar plates and incubated at 37°C for 5 h. At 5 h, the diameter of bacterial spread extending from the site of aEPEC inoculation was measured. Each isolate was assayed on 2 to 3 plates per experiment, and experiments were repeated 3 times. Isolates were considered nonmotile when the diameter of spread was <6 mm, poorly motile when 6 to 9 mm, moderately motile when 9 to 11 mm, and highly motile when >11 mm.

Fluorescence actin-staining test. *In vitro* adherence of kitten aEPEC was performed using HEp-2 cell (ATCC CCL23) monolayers. HEp-2 cells were cultured at 37°C and 5% CO_2 in Eagle's minimum essential medium supplemented with 10% fetal bovine serum and seeded onto 4-well chamber

slides (Nunc Lab-Tek II; Thermo Fisher Scientific, Waltham, MA). HEp-2 monolayers were utilized for adhesion experiments at an estimated confluence of 60% (~10^5 cells per monolayer). Fourteen kitten aEPEC isolates, tEPEC E2348/69, and *E. coli* (ATCC 25922; ATCC, Manassas, VA) were incubated overnight at 37°C and 120 rpm in LB, diluted based on OD_{600} to a concentration of 10^8 CFU/ml, and then inoculated onto HEp-2 monolayers at a multiplicity of infection (MOI) of 10:1. Each isolate was assayed on triplicate monolayers. Infected and medium-only monolayers were incubated at 37°C in 5% CO_2 for a total of 6 h, with nonadhered bacteria being removed after 3 h via washing of the monolayers. Following the 6 h incubation, monolayers were washed with sterile PBS and fixed with 3.7% formalin in PBS (pH 7.0) for 15 min. Monolayers were washed with PBS, permeabilized with 0.5% Triton X-100 for 5 min, and then washed three times with PBS. For visualization of actin aggregation, Acti-stain 488 phalloidin (Cytoskeleton, Inc., Denver, CO) was added to each monolayer, and slides were incubated for 30 min in a humidified chamber protected from light. Following staining, monolayers were washed three times with PBS, and coverslips were applied using Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Labs, Burlingame, CA). Slides were viewed on a Leica DM5000B fluorescence microscope. Following fluorescence imaging, coverslips were removed and monolayers were fixed, stained with eosin and methylene blue (Kwik-Diff; Thermo Scientific, Kalamazoo, MI), and viewed on a traditional bright-field microscope.

Statistical analysis. Data were tested for normal distribution using the Kolmogorov-Smirnov test and for equal variance using the Leven median test and analyzed using parametric or nonparametric statistics as appropriate. Significant differences in distributions of observations between aEPEC isolates were examined using Fisher's exact test and odds ratios. Differences in the mean or median values of continuous data were analyzed using Student's *t* tests, one-way ANOVAs, or Mann-Whitney rank sum tests. Analyses were performed using commercial software (SigmaPlot 12; Systat Software, Inc., San Jose, CA, and Prism, GraphPad Software, San Diego, CA). Data were considered significant when the *P* value was <0.05.

Data availability. The complete genome assemblies of kitten aEPEC genomes sequenced in this study have been deposited in GenBank under the accession numbers VXDG00000000 to VXDR00000000 (Table 1).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.4 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.1 MB.

ACKNOWLEDGMENTS

Research reported in this publication was supported by a grant from the Winn Feline Foundation (grant number W14-035). T.H.H. and D.A.R. were supported by grant U19 AI110820 from the National Institutes of Health. V.E.W. was supported by a Ruth L. Kirschstein National Research Service Award through the National Institutes of Health (NIH) under grant number T32OD011130 and as part of North Carolina State University's Comparative Medicine and Translational Research Training Program. J.R.E. was supported in part by K08AI108794 from the National Institutes of Health.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

We thank James B. Kaper, Chitrita DebRoy, Mitsu Suyemoto, and Anna Rogers for advice and technical assistance.

Study conception and design, Jody L. Gookin, Victoria E. Watson, Megan E. Jacob, Johanna R. Elfenbein, David A. Rasko, and Stephen H. Stauffer; Acquisition of data, Victoria E. Watson, Tracy H. Hazen, and Stephen H. Stauffer; Analysis and interpretation of data, Jody L. Gookin, Victoria E. Watson, Tracy H. Hazen, and David A. Rasko; Drafting of Manuscript, Jody L. Gookin, Victoria E. Watson, Tracy H. Hazen, David A. Rasko, Megan E. Jacob, Johanna R. Elfenbein, and Stephen H. Stauffer; Methodology, Jody L. Gookin, Victoria E. Watson, Tracy H. Hazen, David A. Rasko, Megan E. Jacob, Johanna R. Elfenbein, and Stephen H. Stauffer.

REFERENCES

1. Kirk MD, Pires SM, Black RE, Caipo M, Crump JA, Devleesschauwer B, Dopfer D, Fazil A, Fischer-Walker CL, Hald T, Hall AJ, Keddy KH, Lake RJ, Lanata CF, Torgerson PR, Havelaar AH, Angulo FJ. 2015. World Health Organization estimates of the global and regional disease burden of 22

foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. PLoS Med 12:e1001921. https://doi.org/10.1371/journal.pmed.1001921.
2. Liu J, Platts-Mills JA, Juma J, Kabir F, Nkeze J, Okoi C, Operario DJ, Uddin J, Ahmed S, Alonso PL, Antonio M, Becker SM, Blackwelder WC, Breiman RF, Faruque AS, Fields B, Gratz J, Haque R, Hossain A, Hossain MJ, Jarju S, Qamar F, Iqbal NT, Kwambana B, Mandomando I, McMurry TL, Ochieng C, Ochieng JB, Ochieng M, Onyango C, Panchalingam S, Kalam A, Aziz F, Qureshi S, Ramamurthy T, Roberts JH, Saha D, Sow SO, Stroup SE, Sur D, Tamboura B, Taniuchi M, Tennant SM, Toema D, Wu Y, Zaidi A, Nataro JP, Kotloff KL, Levine MM, Houpt ER. 2016. Use of quantitative molecular diagnostic methods to identify causes of diarrhoea in children: a reanalysis of the GEMS case-control study. Lancet 388:1291–1301. https://doi.org/10.1016/S0140-6736(16)31529-X.
3. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, Breiman RF, Faruque AS, Zaidi AK, Saha D, Alonso PL, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ochieng JB, Omore R, Oundo JO, Hossain A, Das SK, Ahmed S, Qureshi S, Quadri F, Adegbola RA, Antonio M, Hossain MJ, Akinsola A, Mandomando I, Nhampossa T, Acacio S, Biswas K, O'Reilly CE, Mintz ED, Berkeley LY, Muhsen K, Sommerfelt H, Robins-Browne RM, Levine MM. 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382:209–222. https://doi.org/10.1016/S0140-6736(13)60844-2.
4. Pires SM, Fischer-Walker CL, Lanata CF, Devleesschauwer B, Hall AJ, Kirk MD, Duarte AS, Black RE, Angulo FJ. 2015. Aetiology-specific estimates of the global and regional incidence and mortality of diarrhoeal diseases commonly transmitted through food. PLoS One 10:e0142927. https://doi.org/10.1371/journal.pone.0142927.
5. Jerse AE, Yu J, Tall BD, Kaper JB. 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc Natl Acad Sci U S A 87:7839–7843. https://doi.org/10.1073/pnas.87.20.7839.
6. McNamara BP, Koutsouris A, O'Connell CB, Nougayrede JP, Donnenberg MS, Hecht G. 2001. Translocated EspF protein from enteropathogenic *Escherichia coli* disrupts host intestinal barrier function. J Clin Invest 107:621–629. https://doi.org/10.1172/JCI11138.
7. Simonovic I, Rosenberg J, Koutsouris A, Hecht G. 2000. Enteropathogenic *Escherichia coli* dephosphorylates and dissociates occludin from intestinal epithelial tight junctions. Cell Microbiol 2:305–315. https://doi.org/10.1046/j.1462-5822.2000.00055.x.
8. Dean P, Kenny B. 2004. Intestinal barrier dysfunction by enteropathogenic *Escherichia coli* is mediated by two effector molecules and a bacterial surface protein. Mol Microbiol 54:665–675. https://doi.org/10.1111/j.1365-2958.2004.04308.x.
9. Thanabalasuriar A, Koutsouris A, Weflen A, Mimee M, Hecht G, Gruenheid S. 2010. The bacterial virulence factor NleA is required for the disruption of intestinal tight junctions by enteropathogenic *Escherichia coli*. Cell Microbiol 12:31–41. https://doi.org/10.1111/j.1462-5822.2009.01376.x.
10. Tomson FL, Viswanathan VK, Kanack KJ, Kanteti RP, Straub KV, Menet M, Kaper JB, Hecht G. 2005. Enteropathogenic *Escherichia coli* EspG disrupts microtubules and in conjunction with Orf3 enhances perturbation of the tight junction barrier. Mol Microbiol 56:447–464. https://doi.org/10.1111/j.1365-2958.2005.04571.x.
11. Shaw RK, Smollett K, Cleary J, Garmendia J, Straatman-Iwanowska A, Frankel G, Knutton S. 2005. Enteropathogenic *Escherichia coli* type III effectors EspG and EspG2 disrupt the microtubule network of intestinal epithelial cells. Infect Immun 73:4385–4390. https://doi.org/10.1128/IAI.73.7.4385-4390.2005.
12. Dean P, Maresca M, Schuller S, Phillips AD, Kenny B. 2006. Potent diarrheagenic mechanism mediated by the cooperative action of three enteropathogenic *Escherichia coli*-injected effector proteins. Proc Natl Acad Sci U S A 103:1876–1881. https://doi.org/10.1073/pnas.0509451103.
13. Shifflett DE, Clayburgh DR, Koutsouris A, Turner JR, Hecht GA. 2005. Enteropathogenic *E. coli* disrupts tight junction barrier function and *structure in vivo*. Lab Invest 85:1308–1324. https://doi.org/10.1038/labinvest.3700330.
14. Gill RK, Borthakur A, Hodges K, Turner JR, Clayburgh DR, Saksena S, Zaheer A, Ramaswamy K, Hecht G, Dudeja PK. 2007. Mechanism underlying inhibition of intestinal apical Cl/OH exchange following infection with enteropathogenic *E. coli*. J Clin Invest 117:428–437. https://doi.org/10.1172/JCI29625.
15. Hecht G, Hodges K, Gill RK, Kear F, Tyagi S, Malakooti J, Ramaswamy K, Dudeja PK. 2004. Differential regulation of Na^+/H^+ exchange isoform activities by enteropathogenic *E. coli* in human intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 287:G370–8. https://doi.org/10.1152/ajpgi.00432.2003.
16. Afset JE, Bevanger L, Romundstad P, Bergh K. 2004. Association of atypical enteropathogenic *Escherichia coli* (EPEC) with prolonged diarrhoea. J Med Microbiol 53:1137–1144. https://doi.org/10.1099/jmm.0.45719-0.
17. Nguyen RN, Taylor LS, Tauschek M, Robins-Browne RM. 2006. Atypical enteropathogenic *Escherichia coli* infection and prolonged diarrhea in children. Emerg Infect Dis 12:597–603. https://doi.org/10.3201/eid1204.051112.
18. Vernacchio L, Vezina RM, Mitchell AA, Lesko SM, Plaut AG, Acheson DW. 2006. Diarrhea in American infants and young children in the community setting: incidence, clinical presentation and microbiology. Pediatr Infect Dis J 25:2–7. https://doi.org/10.1097/01.inf.0000195623.57945.87.
19. Aslani MM, Alikhani MY. 2011. Molecular and phenotypic characterization of atypical enteropathogenic *Escherichia coli* serotypes isolated from children with and without diarrhea. J Microbiol Immunol Infect 44:27–32. https://doi.org/10.1016/j.jmii.2011.01.006.
20. Donnenberg MS, Hazen TH, Farag TH, Panchalingam S, Antonio M, Hossain A, Mandomando I, Ochieng JB, Ramamurthy T, Tamboura B, Zaidi A, Levine MM, Kotloff K, Rasko DA, Nataro JP. 2015. Bacterial factors associated with lethal outcome of enteropathogenic *Escherichia coli* infection: genomic case-control studies. PLoS Negl Trop Dis 9:e0003791. https://doi.org/10.1371/journal.pntd.0003791.
21. Hazen TH, Donnenberg MS, Panchalingam S, Antonio M, Hossain A, Mandomando I, Ochieng JB, Ramamurthy T, Tamboura B, Qureshi S, Quadri F, Zaidi A, Kotloff KL, Levine MM, Barry EM, Kaper JB, Rasko DA, Nataro JP. 2016. Genomic diversity of EPEC associated with clinical presentations of differing severity. Nat Microbiol 1:15014. https://doi.org/10.1038/nmicrobiol.2015.14.
22. Ingle DJ, Tauschek M, Edwards DJ, Hocking DM, Pickard DJ, Azzopardi KI, Amarasena T, Bennett-Wood V, Pearson JS, Tamboura B, Antonio M, Ochieng JB, Oundo J, Mandomando I, Qureshi S, Ramamurthy T, Hossain A, Kotloff KL, Nataro JP, Dougan G, Levine MM, Robins-Browne RM, Holt KE. 2016. Evolution of atypical enteropathogenic *E. coli* by repeated acquisition of LEE pathogenicity island variants. Nat Microbiol 1:15010. https://doi.org/10.1038/nmicrobiol.2015.10.
23. Contreras CA, Ochoa TJ, Ruiz J, Lacher DW, Durand D, DebRoy C, Lanata CF, Cleary TG. 2012. Genetic diversity of locus of enterocyte effacement genes of enteropathogenic *Escherichia coli* isolated from Peruvian children. J Med Microbiol 61:1114–1120. https://doi.org/10.1099/jmm.0.045443-0.
24. Mercado EH, Piscoche C, Contreras C, Durand D, Riveros M, Ruiz J, Ochoa TJ. 2016. Pathogenicity Island O-122 in enteropathogenic *Escherichia coli* strains is associated with diarrhea severity in children from Lima Peru. Int J Med Microbiol 306:231–236. https://doi.org/10.1016/j.ijmm.2016.05.005.
25. Vieira MA, Salvador FA, Silva RM, Irino K, Vaz TM, Rockstroh AC, Guth BE, Gomes TA. 2010. Prevalence and characteristics of the O122 pathogenicity island in typical and atypical enteropathogenic *Escherichia coli* strains. J Clin Microbiol 48:1452–1455. https://doi.org/10.1128/JCM.01944-09.
26. Afset JE, Bruant G, Brousseau R, Harel J, Anderssen E, Bevanger L, Bergh K. 2006. Identification of virulence genes linked with diarrhea due to atypical enteropathogenic *Escherichia coli* by DNA microarray analysis and PCR. J Clin Microbiol 44:3703–3711. https://doi.org/10.1128/JCM.00429-06.
27. USDA. 2008. Dairy 2007 part II: changes in the US dairy cattle industry, 1991–2007, p 57–61. USDA-APHIS-VS, CEAH, Fort Collins, CO.
28. USDA. 2015. Swine 2012 part II: reference of swine health and health management in the United States, 2012. USDA-APHIS-VS, CEAH, Fort Collins, CO.
29. Ghosh A, Borst L, Stauffer SH, Suyemoto M, Moisan P, Zurek L, Gookin JL. 2013. Mortality in kittens is associated with a shift in ileum mucosa-associated enterococci from *Enterococcus hirae* to biofilm-forming *Enterococcus faecalis* and adherent *Escherichia coli*. J Clin Microbiol 51:3567–3578. https://doi.org/10.1128/JCM.00481-13.
30. Kolenda R, Burdukiewicz M, Schierack P. 2015. A systematic review and meta-analysis of the epidemiology of pathogenic *Escherichia coli* of calves and the role of calves as reservoirs for human pathogenic *E. coli*. Front Cell Infect Microbiol 5:23. https://doi.org/10.3389/fcimb.2015.00023.
31. Moxley RA, Francis DH. 1986. Natural and experimental infection with an attaching and effacing strain of *Escherichia coli* in calves. Infect Immun 53:339–346. https://doi.org/10.1128/IAI.53.2.339-346.1986.
32. Krause G, Zimmermann S, Beutin L. 2005. Investigation of domestic

animals and pets as a reservoir for intimin$^-$ (*eae*) gene positive *Escherichia coli* types. Vet Microbiol 106:87–95. https://doi.org/10.1016/j.vetmic.2004.11.012.
33. Martins FH, Guth BE, Piazza RM, Elias WP, Leao SC, Marzoa J, Dahbi G, Mora A, Blanco M, Blanco J, Pelayo JS. 2016. Lambs are an important source of atypical enteropathogenic *Escherichia coli* in southern Brazil. Vet Microbiol 196:72–77. https://doi.org/10.1016/j.vetmic.2016.10.009.
34. Watson VE, Jacob ME, Flowers JR, Strong SJ, DebRoy C, Gookin JL. 2017. Association of atypical enteropathogenic *Escherichia coli* with diarrhea and related mortality in kittens. J Clin Microbiol 55:2719–2735. https://doi.org/10.1128/JCM.00403-17.
35. Alikhani MY, Mirsalehian A, Aslani MM. 2006. Detection of typical and atypical enteropathogenic *Escherichia coli* (EPEC) in Iranian children with and without diarrhoea. J Med Microbiol 55:1159–1163. https://doi.org/10.1099/jmm.0.46539-0.
36. Watson VE, Jacob ME, Bruno-Barcena JM, Amirsultan S, Stauffer SH, Piqueras VO, Frias R, Gookin JL. 2019. Influence of the intestinal microbiota on disease susceptibility in kittens with experimentally-induced carriage of atypical enteropathogenic *Escherichia coli*. Vet Microbiol 231:197–206. https://doi.org/10.1016/j.vetmic.2019.03.020.
37. Johnson TJ, Nolan LK. 2009. Pathogenomics of the virulence plasmids of *Escherichia coli*. Microbiol Mol Biol Rev 73:750–774. https://doi.org/10.1128/MMBR.00015-09.
38. Hazen TH, Sahl JW, Fraser CM, Donnenberg MS, Scheutz F, Rasko DA. 2013. Refining the pathovar paradigm via phylogenomics of the attaching and effacing *Escherichia coli*. Proc Natl Acad Sci U S A 110:12810–-12815. https://doi.org/10.1073/pnas.1306836110.
39. Vieira MA, Dias RCB, Dos Santos LF, Rall VLM, Gomes TAT, Hernandes RT. 2019. Diversity of strategies used by atypical enteropathogenic *Escherichia coli* to induce attaching and effacing lesion in epithelial cells. J Med Microbiol 68:940–951. https://doi.org/10.1099/jmm.0.000998.
40. Goffaux F, China B, Janssen L, Mainil J. 2000. Genotypic characterization of enteropathogenic *Escherichia coli* (EPEC) isolated in Belgium from dogs and cats. Res Microbiol 151:865–871. https://doi.org/10.1016/s0923-2508(00)01153-0.
41. Afset JE, Anderssen E, Bruant G, Harel J, Wieler L, Bergh K. 2008. Phylogenetic backgrounds and virulence profiles of atypical enteropathogenic *Escherichia coli* strains from a case-control study using multilocus sequence typing and DNA microarray analysis. J Clin Microbiol 46:2280–2290. https://doi.org/10.1128/JCM.01752-07.
42. Morato EP, Leomil L, Beutin L, Krause G, Moura RA, Pestana de Castro AF. 2009. Domestic cats constitute a natural reservoir of human enteropathogenic *Escherichia coli* types. Zoonoses Public Health 56:229–237. https://doi.org/10.1111/j.1863-2378.2008.01190.x.
43. Tennant SM, Tauschek M, Azzopardi K, Bigham A, Bennett-Wood V, Hartland EL, Qi W, Whittam TS, Robins-Browne RM. 2009. Characterisation of atypical enteropathogenic *E. coli* strains of clinical origin. BMC Microbiol 9:117. https://doi.org/10.1186/1471-2180-9-117.
44. Rasko DA, Myers GS, Ravel J. 2005. Visualization of comparative genomic analyses by BLAST score ratio. BMC Bioinformatics 6:2. https://doi.org/10.1186/1471-2105-6-2.
45. Sahl JW, Caporaso JG, Rasko DA, Keim P. 2014. The large-scale blast score ratio (LS-BSR) pipeline: a method to rapidly compare genetic content between bacterial genomes. PeerJ 2:e332. https://doi.org/10.7717/peerj.332.
46. Rocha SP, Abe CM, Sperandio V, Bando SY, Elias WP. 2011. Atypical enteropathogenic *Escherichia coli* that contains functional locus of enterocyte effacement genes can be attaching-and-effacing negative in cultured epithelial cells. Infect Immun 79:1833–1841. https://doi.org/10.1128/IAI.00693-10.
47. Bai L, Schuller S, Whale A, Mousnier A, Marches O, Wang L, Ooka T, Heuschkel R, Torrente F, Kaper JB, Gomes TA, Xu J, Phillips AD, Frankel G. 2008. Enteropathogenic *Escherichia coli* O125:H6 triggers attaching and effacing lesions on human intestinal biopsy specimens independently of Nck and TccP/TccP2. Infect Immun 76:361–368. https://doi.org/10.1128/IAI.01199-07.
48. Nicklas JL, Moisan P, Stone MR, Gookin JL. 2010. *In situ* molecular diagnosis and histopathological characterization of enteroadherent *Enterococcus hirae* infection in pre-weaning-age kittens. J Clin Microbiol 48:2814–2820. https://doi.org/10.1128/JCM.00916-09.
49. DebRoy C, Maddox C. 2001. Identification of virulence attributes of gastrointestinal *Escherichia coli* isolates of veterinary significance. Anim Health Res Rev 2:129–140. https://doi.org/10.1079/AHRR200131.
50. Schmidt H, Knop C, Franke S, Aleksic S, Heesemann J, Karch H. 1995. Development of PCR for screening of enteroaggregative *Escherichia coli*. J Clin Microbiol 33:701–705. https://doi.org/10.1128/JCM.33.3.701-705.1995.
51. Gunzburg ST, Tornieporth NG, Riley LW. 1995. Identification of enteropathogenic *Escherichia coli* by PCR-based detection of the bundle-forming pilus gene. J Clin Microbiol 33:1375–1377. https://doi.org/10.1128/JCM.33.5.1375-1377.1995.
52. Orskov I, Orskov F, Jann B, Jann K. 1977. Serology, chemistry, and genetics of O and K antigens of *Escherichia coli*. Bacteriol Rev 41:667–710. https://doi.org/10.1128/MMBR.41.3.667-710.1977.
53. Machado J, Grimont F, Grimont PA. 2000. Identification of *Escherichia coli* flagellar types by restriction of the amplified *fliC* gene. Res Microbiol 151:535–546. https://doi.org/10.1016/s0923-2508(00)00223-0.
54. Zimin AV, Marcais G, Puiu D, Roberts M, Salzberg SL, Yorke JA. 2013. The MaSuRCA genome assembler. Bioinformatics 29:2669–2677. https://doi.org/10.1093/bioinformatics/btt476.
55. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, Karch H, Reeves PR, Maiden MC, Ochman H, Achtman M. 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol Microbiol 60:1136–1151. https://doi.org/10.1111/j.1365-2958.2006.05172.x.
56. Carattoli A, Zankari E, Garcia-Fernandez A, Voldby Larsen M, Lund O, Villa L, Moller Aarestrup F, Hasman H. 2014. *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 58:3895–3903. https://doi.org/10.1128/AAC.02412-14.
57. Joensen KG, Tetzschner AM, Iguchi A, Aarestrup FM, Scheutz F. 2015. Rapid and easy *in silico* serotyping of *Escherichia coli* isolates by use of whole-genome sequencing data. J Clin Microbiol 53:2410–2426. https://doi.org/10.1128/JCM.00008-15.
58. Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK, Lago BA, Dave BM, Pereira S, Sharma AN, Doshi S, Courtot M, Lo R, Williams LE, Frye JG, Elsayegh T, Sardar D, Westman EL, Pawlowski AC, Johnson TA, Brinkman FS, Wright GD, McArthur AG. 2017. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. Nucleic Acids Res 45:D566–D573. https://doi.org/10.1093/nar/gkw1004.
59. Hazen TH, Nagaraj S, Sen S, Permala-Booth J, Del Canto F, Vidal R, Barry EM, Bitoun JP, Chen WH, Tennant SM, Rasko DA. 2019. Genome and functional characterization of colonization factor antigen I- and CS6-encoding heat-stable enterotoxin-only enterotoxigenic *Escherichia coli* reveals lineage and geographic variation. mSystems 4:e00329-18. https://doi.org/10.1128/mSystems.00329-18.
60. Sahl JW, Beckstrom-Sternberg SM, Babic-Sternberg JS, Gillece JD, Hepp CM, Auerbach RK, Tembe W, Wagner DM, Keim PS, Pearson T. 2015. The *in silico* genotyper (ISG): an open-source pipeline to rapidly identify and annotate nucleotide variants for comparative genomics applications. bioRxiv https://www.biorxiv.org/content/10.1101/015578v1.
61. Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690. https://doi.org/10.1093/bioinformatics/btl446.
62. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739. https://doi.org/10.1093/molbev/msr121.
63. Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680. https://doi.org/10.1093/nar/22.22.4673.
64. Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120. https://doi.org/10.1007/BF01731581.
65. Deacon V, Dziva F, van Diemen PM, Frankel G, Stevens MP. 2010. Efa-1/LifA mediates intestinal colonization of calves by enterohaemorrhagic *Escherichia coli* O26:H$-$ in a manner independent of glycosyltransferase and cysteine protease motifs or effects on type III secretion. Microbiology (Reading) 156:2527–2536. https://doi.org/10.1099/mic.0.039685-0.
66. Klapproth JM, Sasaki M, Sherman M, Babbin B, Donnenberg MS, Fernandes PJ, Scaletsky IC, Kalman D, Nusrat A, Williams IR. 2005. Citrobacter rodentium lifA/efa1 is essential for colonic colonization and crypt cell hyperplasia in vivo. Infect Immun 73:1441–1451. https://doi.org/10.1128/IAI.73.3.1441-1451.2005.

67. Badea L, Doughty S, Nicholls L, Sloan J, Robins-Browne RM, Hartland EL. 2003. Contribution of Efa1/LifA to the adherence of enteropathogenic *Escherichia coli* to epithelial cells. Microb Pathog 34:205–215. https://doi.org/10.1016/s0882-4010(03)00026-3.
68. Kobayashi H, Shimada J, Nakazawa M, Morozumi T, Pohjanvirta T, Pelkonen S, Yamamoto K. 2001. Prevalence and characteristics of Shiga toxin-producing *Escherichia coli* from healthy cattle in Japan. Appl Environ Microbiol 67:484–489. https://doi.org/10.1128/AEM.67.1.484-489.2001.
69. Kyaw CM, De Araujo CR, Lima MR, Gondim EG, Brigido MM, Giugliano LG. 2003. Evidence for the presence of a type III secretion system in diffusely adhering *Escherichia coli* (DAEC). Infect Genet Evol 3:111–117. https://doi.org/10.1016/s1567-1348(03)00008-x.
70. Sharma VK, Zuerner RL. 2004. Role of *hha* and *ler* in transcriptional regulation of the *esp* operon of enterohemorrhagic *Escherichia coli* O157:H7. J Bacteriol 186:7290–7301. https://doi.org/10.1128/JB.186.21.7290-7301.2004.
71. Narimatsu H, Ogata K, Makino Y, Ito K. 2010. Distribution of non-locus of enterocyte effacement pathogenic island-related genes in *Escherichia coli* carrying eae from patients with diarrhea and healthy individuals in Japan. J Clin Microbiol 48:4107–4114. https://doi.org/10.1128/JCM.00677-10.
72. O'Toole GA. 2011. Microtiter dish biofilm formation assay. J Vis Exp 47:e2437. https://doi.org/10.3791/2437.
73. Levine MM, Bergquist EJ, Nalin DR, Waterman DH, Hornick RB, Young CR, Sotman S. 1978. *Escherichia coli* strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. Lancet 1:1119–1122. https://doi.org/10.1016/s0140-6736(78)90299-4.
74. Maes A, Gracia C, Brechemier D, Hamman P, Chatre E, Lemelle L, Bertin PN, Hajnsdorf E. 2013. Role of polyadenylation in regulation of the flagella cascade and motility in *Escherichia coli*. Biochimie 95:410–418. https://doi.org/10.1016/j.biochi.2012.10.017.
75. Young GM, Smith MJ, Minnich SA, Miller VL. 1999. The *Yersinia enterocolitica* motility master regulatory operon, flhDC, is required for flagellin production, swimming motility, and swarming motility. J Bacteriol 181:2823–2833. https://doi.org/10.1128/JB.181.9.2823-2833.1999.